Human Papillomavirus E6 Triggers Upregulation of the Antiviral and Cancer Genomic DNA Deaminase APOBEC3B

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ABSTRACT Several recent studies have converged upon the innate immune DNA cytosine deaminase APOBEC3B (A3B) as a significant source of genomic uracil lesions and mutagenesis in multiple human cancers, including those of the breast, head/neck, cervix, bladder, lung, ovary, and other tissues. A3B is upregulated in these tumor types relative to normal tissues, but the mechanism is unclear. Because A3B also has antiviral activity in multiple systems and is a member of the broader innate immune response, we tested the hypothesis that human papillomavirus (HPV) infection causes A3B upregulation. We found that A3B mRNA expression and enzymatic activity were upregulated following transfection of a high-risk HPV genome and that this effect was abrogated by inactivation of E6. Transduction experiments showed that the E6 oncoprotein alone was sufficient to cause A3B upregulation, and a panel of high-risk E6 proteins triggered higher A3B levels than did a panel of low-risk or noncancer E6 proteins. Knockdown experiments in HPV-positive cell lines showed that endogenous E6 is required for A3B upregulation. Analyses of publicly available head/neck cancer data further support this relationship, as A3B levels are higher in HPV-positive cancers than in HPV-negative cancers. Taken together with the established role for high-risk E6 in functional inactivation of TP53 and published positive correlations in breast cancer between A3B expression and TP53 inactivation, our studies suggest a model in which high-risk HPV E6, possibly through functional inactivation of TP53, causes derepression of A3B gene transcription. This would lead to a mutator phenotype that explains the observed cytosine mutation biases in HPV-positive head/neck and cervical cancers.

IMPORTANCE The innate immune DNA cytosine deaminase APOBEC3B (A3B) accounts for a large proportion of somatic mutations in cervical and head/neck cancers, but nothing is known about the mechanism responsible for its upregulation in these tumor types. Almost all cervical carcinomas and large proportions of head/neck tumors are caused by human papillomavirus (HPV) infection. Here, we establish a mechanistic link between HPV infection and A3B upregulation. The E6 oncoprotein of high-risk, but not low-risk, HPV types triggers A3B upregulation, supporting a model in which high-risk HPV E6, possibly through functional inactivation of TP53, causes derepression of A3B gene transcription and elevated A3B enzyme levels. This virus-induced mutator phenotype provides a mechanistic explanation for A3B signature mutations observed in HPV-positive head/neck and cervical carcinomas and may also help to account for the preferential cancer predisposition caused by high-risk HPV isolates.


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enzymes for 5’ TC and 5’ CC substrates). Remarkably, a single HIV-1 protein, Vif, is able to physically bind and counteract all four of these restriction factors by recruiting an E3 ubiquitin ligase complex to target them for proteasomal degradation. Based on evidence for restriction and/or G-to-A mutation, other potentially A3-susceptible human viruses include adeno-associated virus (AAV), Epstein-Barr virus (EBV), hepatitis B virus (HBV), herpes simplex virus 1 (HSV-1), human T-cell lymphotropic virus (HTLV), and, most relevant to this study, human papillomavirus (HPV) (9–13; reviewed in reference 14).

HPV is an ~8-kb double-stranded DNA virus that replicates in the nucleus of mucosal or cutaneous keratinocytes (reviewed in reference 15). Over 170 HPV types have been identified thus far, and these can be classified into high- and low-risk groups based on carcinogenic risk (16–18). HPV infection is necessary but not sufficient for the development of cervical cancer, and it is also strongly associated with other anogenital and a growing subset of head/neck squamous cancers (19, 20). The viral oncoproteins E6 and E7 are invariably expressed in HPV-positive cancers (21, 22), and the expression of these proteins from high-risk isolates is sufficient to immortalize human keratinocytes (23, 24). The most critical functions of E6 and E7, respectively, are thought to be functional inactivation of tumor suppressors TP53 and RB (25–28).

Recently, the enzymatic activity of A3B has been implicated as a major source of mutagenesis in multiple human cancers (29–37). A3B is a nuclear enzyme and the only detectable source of single-stranded DNA cytosine deaminase activity in multiple cancer cell lines (29, 32). A3B mRNA levels are upregulated in many cancer types, including those of the breast, bladder, cervix, lung, head/neck, and ovary (29–32). The trinucleotide preference of A3B (5’ TCA and 5’ TCG) is highly enriched in the mutation spectrum of these cancer types (29–32). Moreover, positive correlations are evident between A3B mRNA levels and somatic mutation loads (30–32). Interestingly, head/neck and cervical cancers are among the tumor types displaying the highest A3B expression levels and cytosine mutational loads in A3B-preferred trinucleotide contexts (30, 31). Overall, a compelling case has been made for A3B mutagenesis in multiple human cancers.

Given the fact that A3B is expressed at low levels or not at all in most normal tissues (29, 30, 38), a major unresolved question is how it becomes upregulated in cancer. Since A3B is a member of the A3 family of innate immune effector proteins with demonstrated antiviral activities (though not against HIV-1 in T lymphocytes [39, 40]) and given the tendency of HPV-associated cancers of the head/neck and cervix to be among the highest A3B-impacted tumor types, here we test the hypothesis that HPV directly causes A3B mRNA upregulation. Moreover, because E6 is invariably expressed in HPV-positive tumors (21, 22) and A3B upregulation is associated with genetic inactivation of TP53 (29), we tested the specific hypothesis that E6 is the primary trigger of A3B upregulation in virus-positive tumor types.

RESULTS

HPV genomic DNA causes A3B upregulation. We first tested whether high-risk HPV genomes could trigger A3B upregulation. Normal immortalized keratinocytes (NIKS) were transfected with full-length HPV16 or HPV18 genomes. Pools of transfectants were selected and expanded to allow for establishment of the viral genomes as nuclear plasmids and viral gene expression, and then reverse transcription–quantitative PCR (RT-qPCR) was used to quantify A3B mRNA levels. In comparison to a control vector-transfected pool of NIKS established in parallel, A3B mRNA levels were induced significantly by transfection of either HPV16 or HPV18 genomes (Fig. 1A). HPV18 genomic DNA consistently caused higher levels of A3B induction, routinely 5- to 10-fold above the negative control.

To ask whether the effect of HPV genomic DNA is specific to A3B or to a more general antiviral response, RT-qPCR assays were used to quantify expression of all A3 family members (Fig. 1A). Most of these genes, including A3A, A3D, A3H, AID, A1, A2, and A4, were expressed at very low or undetectable levels and not affected by HPV genomic DNA transfection. Two family members, A3F and A3G, were expressed at similar levels in both control and HPV-transfected NIKS. The only exception was A3C, which showed an inverse relationship with higher levels in control-transfected cells and lower levels in HPV-transfected cells, especially with HPV18 genomic DNA. Thus, A3B is the only DNA deaminase family member upregulated at the level of transcription in NIKS harboring HPV genomes. Since there are no commercial antibodies for A3B, the current gold standard for detecting A3B protein levels is by quantifying its functional activity (29, 32). Therefore, we performed single-stranded DNA deaminase assays using protein extracts from the same cells as used for mRNA quantification (Fig. 1B). As expected, protein extracts produced from cells transfected with HPV18 had deaminase activity more than five times higher than that of the transfection control (68% versus 12% substrate deamination, respectively). These results show that transfection of HPV18 genome results in an increase in DNA deaminase activity that is proportional to the increase observed for A3B mRNA.

HPV E6 is sufficient for A3B upregulation. Considering that the viral oncoproteins are invariably expressed in HPV-positive tumors, we next tested for a possible role of E6 in A3B upregulation. NIKS were transfected either with the full-length HPV18 genome or with a full-length HPV18 genome containing a stop codon within the E6 open reading frame (HPV18 E6-STOP) (41). As shown above, transfection with the wild-type HPV18 genome resulted in a significant upregulation of A3B mRNA levels. However, most of this effect was lost upon transfection with the HPV18 genome containing an E6-STOP mutation, indicating that E6 is required for induction of A3B (Fig. 2A). E6 mRNA levels were also reduced likely due to nonsense-mediated decay (Fig. 2B). To reconfirm the correlation between upregulation of A3B mRNA levels and enzymatic activity, DNA deaminase assays were performed using cell extracts. As expected, the DNA cytosine deaminase activity induced by transfection with wild-type HPV18 genome was ablated by inactivation of E6 (Fig. 2C).

To test if expression of E6 is sufficient to induce A3B upregulation, we used a panel of transduced cell lines based on the hTERT-immortalized keratinocyte cell line N/TERT-1. Each line expressed a different E6 protein from a genus alpha high-risk type (HPV16, -18, -33, -45, and -52), a low-risk type (HPV6b and -11), or a type with no known cancer association (HPV2a and -57) (42, 43). Interestingly, only cells expressing high-risk E6 proteins showed significant increases in A3B mRNA levels in comparison to an empty vector control and E6 from low-risk and non-cancer-associated HPV types (P < 0.01; Fig. 3).

We next asked if these results extended to primary keratinocytes. Early-passage human keratinocyte G5-Ep cells were transf-
duced with the same panel of retroviruses expressing E6 from different HPV types. As above, A3B upregulation was induced and high-risk E6 proteins caused higher levels of induction ($P < 0.05$) (see Fig. S1 in the supplemental material). Together, the data with NIKS, N/TERT-1, and early-passage keratinocytes demonstrate that E6 alone, especially from high-risk HPV types, is sufficient to induce A3B upregulation.

E6 is required for endogenous A3B expression in HPV-positive cancer cell lines. To test if endogenous E6 could contribute to upregulation of endogenous A3B, we depleted the HPV early transcript from the HPV16-positive CaSki cell line. Two different small interfering RNAs (siRNAs) were used to interfere with E6 expression. In each instance, the level of E6 depletion was proportional to the decrease in endogenous A3B mRNA levels with an approximately 3-fold reduction in E6 mRNA levels and a corresponding 3-fold reduction in A3B mRNA levels (Fig. 4).

These results indicate that endogenous E6 contributes to upregulation of endogenous A3B.

**Figure 1.** APOBEC3B upregulation by transfection of full-length HPV genomes. (A) Histograms reporting APOBEC family member mRNA levels in NIKS transfected with a full-length HPV16 or HPV18 or a control plasmid (Cont.). Each histogram bar shows the mean expression level of each APOBEC family member normalized to TBP (error bars report standard deviations from triplicate assays). (B) Image of the results of a representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in panel A. The single-stranded DNA substrate was treated with reaction buffer as a negative control (−) and recombinant APOBEC3A as a positive control (+).
proteins did not cause significant bars report standard deviations from triplicate assays). Low-risk/noncancer E6 histogram bar shows the mean noncancer types (HPV2a and -57) or with an empty vector (Cont.). Each types (HPV16, -18, -33, -45, and -52), low-risk types (HPV6b and -11), or levels in N/TERT-1 cells transduced with HPV E6 from different high-risk (error bars report standard deviations from triplicate assays). (C) Image of the results of a representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in panels A and B. The single-stranded DNA substrate was treated with reaction buffer as a negative control (−) and recombinant APOBEC3A as a positive control (+).

acquire RNA-seq counts for 23 of the HPV-positive patients and 69 of the HPV-negative patients. A3B mRNA levels were significantly increased in HPV-positive compared to HPV-negative cancers (P = 0.0006) (Fig. 5A). Notably, a significant increase in A3B expression was evident for the subset of HPV-positive patients with no smoking history (n = 6) compared to corresponding HPV-negative nonsmokers (n = 10; P = 0.0013).

**DISCUSSION**

This is the first study to demonstrate a mechanistic link between HPV infection and upregulation of the DNA cytosine deaminase A3B. Here we show that transfection of the HPV genome triggers A3B upregulation and that E6 expression is required. HPV upregulation is apparent at both mRNA and activity levels. We demonstrate that high-risk E6 alone is sufficient for the induction of A3B in keratinocytes and that continuous expression of E6 is required to maintain higher A3B levels in HPV-positive cancer cell lines.

Finally, analyses of available TCGA data show that A3B levels are higher in head/neck HPV-positive cancers than in HPV-negative cancers. Taken together, these results suggest a model in which high-risk HPV E6 induces A3B gene expression, leading to a mutator phenotype and the observed cytosine mutation biases in HPV-positive head/neck and cervical cancers.

A3B has been strongly implicated in mutagenesis in a wide variety of human cancers (29–37). Given its relatively low expression level in almost all normal tissues (29, 30, 38), a major question is how A3B upregulation occurs in cancer cells. As described here, the E6 oncoprotein provides the first mechanistic link between viral infection and A3B-mediated cancer mutagenesis. Although E6 has numerous functions, we propose a direct model in which high-risk E6 proteins inactivate TP53 and cause derepression of A3B gene transcription. This mechanism has the potential to explain A3B upregulation in HPV-positive cervical and head/neck cancers (and possibly other cancers such as some bladder carcinomas where HPV may also contribute [19, 20, 44]). This connection between TP53 function and A3B upregulation is supported by our previous observation that genetic inactivation of TP53 correlates positively with elevated A3B levels in breast cancer cell lines and primary tumors (29). Therefore, a model involving TP53 inactivation may apply more generally and contribute to tumorigenesis on at least two distinct levels, by elevating levels of DNA damage and mutation through A3B and by preventing the DNA damage response and apoptosis. Additional studies are necessary to distinguish between this model and other, less-direct possibilities such as an association of E6 with cellular PDZ domain proteins, a characteristic that is also shared by genus alpha high-risk E6 proteins (45, 46). These E6 proteins have a PDZ-binding domain that interacts with a number of PDZ targets with a wide array of functions, including cell signaling, polarity determination, and cell proliferation (reviewed in references 47 and 48).

Although E6 and E7 are sufficient to immortalize primary keratinocytes (23, 24), complete cellular transformation also requires the introduction of additional activated oncogenes or extensive periods of cell culture (49–51). These observations strongly suggest that additional somatic mutations are required for transformation (reviewed in reference 52). We hypothesize that E6 expres-

**FIG 2** HPV18 E6 is necessary for APOBEC3B upregulation. (A) A3B and (B) E6 mRNA levels in NIKS transfected with full-length HPV18 (WT), with HPV18 with a stop codon truncating the E6 open reading frame (E6-STOP), or with a control plasmid (Cont.). Each histogram bar shows the mean mRNA expression level normalized to TBP (error bars report standard deviations from triplicate assays). (C) Image of the results of a representative DNA cytosine deaminase assay performed with cell extracts from the same cells as in panels A and B. The single-stranded DNA substrate was treated with reaction buffer as a negative control (−) and recombinant APOBEC3A as a positive control (+).

**FIG 3** Upregulation of APOBEC3B by expression of HPV E6. A3B mRNA levels in N/TERT-1 cells transduced with HPV E6 from different high-risk types (HPV16, -18, -33, -45, and -52), low-risk types (HPV6b and -11), or noncancer types (HPV2a and -57) or with an empty vector (Cont.). Each histogram bar shows the mean A3B expression level normalized to TBP (error bars report standard deviations from triplicate assays). Low-risk/noncancer E6 proteins did not cause significant A3B upregulation compared to high-risk E6 proteins (P < 0.01; two-tailed Student’s t test).

4 mBio mbio.asm.org November/December 2014 Volume 5 Issue 6 e02234-14
sion leads to elevated A3B levels and an increased, but still stochastic, mutational process that leads eventually to transformation. The same deamination process may also explain genomic instability phenotypes previously shown to be inducible by high-risk oncogenes (reviewed in reference 52).

Our data provide evidence that HPV infection causes upregulation of A3B, a phenomenon generally regarded as an innate antiviral response and previously observed for HIV-1 infection of primary T lymphocytes (1, 39, 40). This relationship prompts the additional question of how HPV avoids restriction in the presence of increased A3B activity and constitutive levels of other A3 proteins. Despite the fact that overexpression studies have shown that HPV can be mutated by APOBEC3 (A/C/H) (11, 53), clinical isolates rarely show evidence for hypermutation (11), consistent with HPV-APOBEC3 counteraction or avoidance strategy operating in vivo. Based on precedents with other viruses (notably lentiviruses and foamy viruses reviewed in reference 1), the answer to this question may provide fundamental mechanistic insights into the HPV replication and transmission cycle. Finally, the robust cellular response to HPV infection characterized by A3B upregulation strongly suggests that other viruses may also be able to provoke similar responses. A mechanistic linkage to the innate antiviral response may also help to explain A3B upregulation and genomic mutagenesis observed in other cancers such as those of the lung, bladder, and breast tissues.

MATERIALS AND METHODS

Cell lines. Normal immortal keratinocytes (NIKS; provided by Lynn Allen-Hoffman [54]) were cultured in E medium supplemented with 24 µg/ml adenine, 8.4 µg/ml chola toxin, 10 ng/ml epidermal growth factor (EGF), 400 ng/ml hydrocortisone, 5 µg/ml insulin, 1% penicillin-streptomycin, and 5% fetal bovine serum and grown in the presence of mitomycin C-treated J2-3T3 feeder cells (55).

Human hTERT-immortalized keratinocytes (N/TERT-1) and G5-Ep primary human foreskin keratinocytes (provided by James Rheinwald [56–58]) were cultured in keratinocyte serum-free medium (K-SFM) supplemented with 0.3 mM CaCl2, 0.2 ng/ml EGF, 25 µg/ml bovine pituitary extract, and 1% penicillin-streptomycin.

HPV genome transfections. NIKS were transfected with the HPV genome as described previously (41). Full-length HPV genomes from wild-type HPV16 and HPV18 and from HPV18 containing a stop codon at E6 (E6-STOP) were excised from their bacterial vectors with either BamHI or NcoI and recircularized with T4 DNA ligase (15 U/µl) at a concentration of 8 ng/µl DNA. One day prior to transfection, 3 × 10⁴ NIKS were plated in low-Ca²⁺ incomplete E medium in the absence of J2-3T3 feeders. Cells were transfected with 3 µg of religated HPV and 1.2 µg of a plasmid conferring neomycin resistance (pEGFP-N1) using Effectene (Qiagen). HPV-negative controls were transfected with 1.2 µg of pEGFP-N1 alone. The next day, cells were transferred to a 10-cm dish containing J2-3T3 feeders. Cells were selected for 4 days in the presence of G418 (125 µg/ml for 2 days followed by 250 µg/ml for 2 days). Two to 3 weeks after transfection, colonies were pooled and expanded. Cells were passaged on a weekly basis and were grown until approximately 90% confluent prior to harvesting of total RNA.

Retroviral transductions. N/TERT-1 and G5-Ep primary keratinocytes stably expressing HPV16 E6 have been described elsewhere (42, 43). Cells were transduced with a panel of retroviruses (pMSCV-N-HA-IRESPURO) expressing E6 from genus alpha high-risk types (HPV16, -18, -33, -45, and -52), from low-risk types (HPV6b and -11), or from types with no known cancer association (HPV2a and -57) (42). As a negative control, cells were transduced with the empty vector (pMSCV-N-HA-IRESPURO empty). Cells were selected with puromycin and grown to approximately 30% confluence prior to harvesting of total RNA. Expression differences were assessed using a two-tailed Student t test.

siRNA transfections. CaSki cells were transfected using DharmaFECT 1 (Dharmacon/GE Life Sciences) as described elsewhere (59).

FIG 4 HPV E6 knockdown reduces endogenous A3B expression. E6 mRNA levels (A) and A3B mRNA levels (B) in CaSki cells transfected with siRNA targeting the HPV16 early transcript (#1 or #2) or with a nontargeting control siRNA (Cont.). Each histogram bar shows the mean E6 or A3B expression level relative to TBP (error bars report standard deviations from triplicate assays).

FIG 5 APOBEC3B overexpression in HPV-positive head/neck tumors. (A) A3B mRNA levels in HPV-positive and HPV-negative head/neck cancers (HPV positive, n = 69; HPV negative, n = 23; P = 0.0006). (B) A3B mRNA levels in the subset of patients in panel A reported as never-smokers (HPV positive, n = 6; HPV negative, n = 10; P = 0.0013). Each histogram bar shows the average A3B expression level normalized to TBP, with values derived from TCGA RNA-seq data sets (error bars report the standard deviations).
plexes were used as follows: nontargeting siRNA (Dharmacon/GE Life Sciences D-001810-01) and two custom-designed siRNAs targeting the HPV16 early transcript. SIGLO red transfection indicator (Dharmacon/GE Life Sciences D-001163-00) was used to visualize efficient transfection in a control well. Sequences of the custom siRNAs are as follows: HPV16 #1, CCAACUUGAAGCCAGCAUAUU, and HPV16 #2, GGACA GAGGCCCAUAUCAAUU. siRNAs were used at a final concentration of 40 nM. Cells were harvested at 72 h posttransfection.

Quantification of cellular and viral RNA. Reverse transcription-quantitative PCR (RT-qPCR) was used to measure APOBEC and E6 mRNA levels as described previously (38). Total RNA was isolated using the NucleoSpin RNA kit (Clontech). One microgram from each sample was reverse transcribed with the cDNA Transcriptor reverse transcriptase (Roche; catalog no. 04887301001). Primer and probe sets were as follows: 16E6-F, 5′-GACCAGAGAAGACGTGCA; 16E6-R, 5′-TTGTGGGACTGTCG TCTGATAA; UPL#115; 18E6-F, 5′-ACATGGAAACCATACATACAC TGG; 18E6-R, 5′-TCGTTTTTTCTAATGGTTGTTAATTT; UPL#120. For each condition, qPCRs were performed in triplicate, mRNA expression levels were normalized to those of the housekeeping gene TBP, and the mean and standard deviation were reported.

DNA deaminase activity assays. Degamination reactions were performed at 37°C for 2 h using 16.5 μL of cell extract, 4 pmol of oligonucleotide (5′-ATTATTATTATATCAATTTATTTTTATTTATTTA TTT-fluorescein), 0.025 μM uracil DNA glycosylase (UDG), 2 μM 10× UDG buffer (NEB), and 1.75 μL RNase A. Reaction mixtures were treated with 100 mM NaOH at 95°C for 10 min to achieve complete backbone breakdown. Reaction mixtures were separated on 15% Tris-borate-EDTA (TBE)–agarose gel. Densitometry was performed using ImageQuant TL (GE Healthcare Life Science).

Head and neck cancer data retrieval and analyses. Head/neck cancer data were acquired from The Cancer Genome Atlas (TCGA) (60), and individuals were selected for analysis if HPV status was clear. RNA-seq counts were used to quantify A3B mRNA expression levels and calculate abundance relative to TBP. This metric facilitates cross-comparisons with RT-qPCR data similarly normalized. Expression differences were assessed using a two-tailed Student t test. Results were considered significant if the calculated P value was under 0.05.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02234-14/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.

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